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The soluble guanylate cyclase stimulator riociguat and the soluble guanylate cyclase activator cinaciguat exert no direct effects on contractility and relaxation of cardiac myocytes from normal rats

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Riociguat (PubChem CID: 11304743)
Cinaciguat (PubChem CID: 9808022)
H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (PubChem CID: 1456)
Isoproterenol (PubChem CID: 3779)
Verapamil (PubChem CID: 62969)
8-(4-Chlorophenylthio)-guanosine3′,5′-cyclic monophosphate sodium salt (8-pCPT–cGMP) (PubChem CID: 23679064)

ABSTRACT

In cardiovascular diseases, reduced responsiveness of soluble guanylate cyclase (sGC) to nitric oxide (NO) upon long-term application has led to the development of NO-independent sGC stimulators (heme-dependent) and sGC activators (heme-independent). Any direct inotropic or lusitropic effects of these compounds on isolated cardiac myocytes, however, remain to be elucidated.

Here, we analyzed the dose-dependent effects of clinical relevant concentrations (10^{-10}–10^{-5} M) of the sGC activator cinaciguat and the sGC stimulator riociguat on the contraction, relaxation, and calcium transients of isolated field-stimulated cardiac myocytes from healthy rats. For comparison, we used isoproterenol, which induced a dose-dependent significant increase in cell contractility, relaxation, and calcium transients, verapamil that significantly decreased these parameters (both at 10^{-9}–10^{-5} M) and 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT–cGMP) that induced a negative inotropic effect at 10^{-5} M accompanied by a slight increase in relaxation. In contrast, neither cinaciguat nor riociguat significantly influenced any measured parameters. Furthermore, isoproterenol significantly increased intracellular cAMP levels that were not influenced by cinaciguat or riociguat (all at 10^{-6} M). Otherwise, riociguat and cinaciguat (both at 10^{-6} M) significantly enhanced intracellular cGMP generation. This accumulation was significantly augmented by cinaciguat in the presence of the sGC inhibitor 1 H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 25 μM), whereas ODQ blocked cGMP generation by riociguat. However, blocking of sGC did not influence cell contractility.

Our results demonstrate that, in isolated cardiac myocytes from healthy rats, the increase in cGMP levels induced by cinaciguat and riociguat at clinical relevant concentrations is not associated with acute direct effects on cell contraction and relaxation.

1. Introduction

In many clinical conditions with impaired bioavailability of nitric oxide (NO) – e.g., coronary artery disease – organic nitrates and NO donor compounds are administered to restore the sGC-cGMP signaling cascade (Evgenov et al., 2006). Serious limitations - such as reduced responsiveness of the soluble guanylate cyclase (sGC) to NO following prolonged administration of NO (Evgenov et al., 2006; Pacher et al., 2007; Stasch et al., 2011) - have led to the development of two classes of compounds that amplify the function of sGC in a NO-independent manner: sGC stimulators and sGC activators (Stasch et al., 2011). Here, the action of stimulators depends on the presence of a reduced (ferrous) prosthetic heme, whereas activators preferentially and effectively activate the oxidized/heme-free sGC (Stasch et al., 2011).

The recently discovered sGC stimulator riociguat and the sGC activator cinaciguat had reached the most advanced development stage in both experimental and clinical research. Both have been shown to induce various beneficial effects in various cardiovascular disease models for myocardial infarction, chronic renal failure, arterial and pulmonary hypertension (PH) and chronic heart

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failure (Evgenov et al., 2006; Stasch et al., 2011). For instance: intravenous administration of cinaciguat resulted in a dose-dependent reduction in cardiac pre- and afterload in a canine model of congestive heart failure, accompanied by an increase in cardiac output and renal blood flow without further neurohumoral activation (Boerrigter et al., 2007). Stimulation of sGC with riociguat led to reduced pulmonary arterial pressure in two rodent models of PH and partially reversed right heart hypertrophy and vascular remodeling (Schermuly et al., 2008). Initial clinical studies revealed that treatment with riociguat induced a dose-dependent improvement of pulmonary hemodynamic parameters and cardiac index in patients with PH, but with significant systemic effects and without pulmonary selectivity (Grimminger et al., 2009). Moreover, in patients with PH caused by systolic left ventricular dysfunction, riociguat improved cardiac index as well as pulmonary and systemic vascular resistance (Bonderman et al., 2013).

Given the broad spectrum of cardiovascular diseases in which treatment with riociguat and cinaciguat may be applicable, comprehensive knowledge of the mechanism of the cardiovascular effects of these compounds is necessary. Moreover, in consideration of the fact that positive inotropic cAMP-enhancing drugs such as phosphodiesterase-3 inhibitors have been related to adverse outcome in heart failure patients (Packer et al., 1991) it is essential to evaluate potential detrimental effects.

Until now, no data have been reported on direct inotropic and lusitropic effects of cinaciguat and riociguat or of related compounds on isolated cardiac myocytes (Hammond and Balligand, 2012). The present study was therefore designed to ascertain potential dose-dependent direct effects of both compounds on contractility and relaxation of isolated rat cardiac myocytes, as well as determining their effects on cAMP and cGMP production. Compared to isolated perfused hearts, in which changes in coronary circulation may be involved in modulation of myocardial contraction and relaxation, an experimental setup with isolated cardiac myocytes entirely avoids simultaneous effects on coronary circulation.

2. Materials and methods

2.1. Ethics statement

All procedures involving experimental animals were performed in accordance with the directive 2010/63/EU on the protection of animals used for scientific purposes and the German animal protection act.

2.2. Isolation of rat cardiac myocytes and measurement of calcium transients and cell shortening

Isolation of ventricular cardiac myocytes from adult rats as well as measurements of intracellular calcium transients and systolic cell shortening were performed as described earlier (Felix et al., 2002; Kubin et al., 1999; Staudt et al., 2004). In brief, isolated hearts from adult female Wistar rats were perfused with a modified oxygenated Ca^{2+}-free Krebs–Henseleit buffer (110 mM NaCl, 2.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM HEPES, and 11 mM glucose, pH 7.4 at room temperature). After collagenase digestion, the extracted ventricles were mechanically minced and Ca^{2+} was restored by centrifugation and resuspension of the cells in incrementally increased Ca^{2+} -solution. For cell adhesion, isolated cardiac myocytes were re-suspended in a Krebs-Henseleit buffer (117 mM NaCl, 2.8 mM KCl, 0.6 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 20 mM glucose, 10 mM HEPES, pH 7.3) and plated on laminin-coated four-well chamber glass slides in a concentration of 5×10^4 cells per chamber for 60 min. Afterwards, the cells were loaded with the fluorescent Ca^{2+} -indicator Fura 2-AM in the presence of 0.5% dimethyl sulfoxide (DMSO) for 10 min.

Cells were field-stimulated by a custom-built stimulator through bipolar silver-chloride electrodes at a frequency of 1 Hz for a duration of 5 ms. Cell shortening and Ca^{2+}-dependent changes in Fura-2-AM fluorescence of single-ventricular myocytes were simultaneously measured by a video-edge detection system (IonOptix, Milton, USA) connected to a standard inverse fluorescent microscope (Leica, Wetzlar, Germany), with employment of a dual-excitation, single-emission fluorescence photomultiplier system (IonOptix, Milton, USA). Emitted light was detected between 480 nm and 520 nm by a photomultiplier tube. Changes in calcium transients were calculated as peak systolic relative fluorescence units minus diastolic relative fluorescence units without the calibration effort, owing to uncertain subcellular compartmentalization of the probes. Contractile properties were analyzed using maximum cell length (μm), minimum cell length (μm), absolute cell shortening (μm), cell contraction velocity [peak DeltaL/Delta(t) shortening] (μm/s), and cell relaxation velocity [peak Delta*L*/Delta(*t*) relaxation] (μm/s) (Bramlage et al., 2001). The various parameters were determined with the aid of the IonOptix software IonWizzard 6.1.

2.3. Experimental design for measurements of cell contraction and calcium transients

For each concentration at least 5 different cardiac myocytes from at least 4 different cardiac myocyte preparations were measured. Only rod-shaped cells that showed clear striations and no spontaneous contractions were used.

After a 2-min equilibration period with 1 Hz stimulation, the basal contraction and calcium transients of the monitored cell were measured (baseline), followed by addition of the substance to be investigated to the Krebs-Henseleit buffer. For calculation of changes in systolic cell shortening and calcium transients, cell length and Fura 2-AM fluorescence of the monitored cell were measured again after 5 min. For each cell at each point (baseline, 5 min) the mean value out of 10 single contractions was used for further calculations.

2.3.1. Concentration range of the investigated compounds

To investigate dose-dependent effects, rat cardiac myocytes were incubated with the sGC activator cinaciguat or the sGC stimulator riociguat – both at concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. For assessment of positive inotropic, β-adrenoceptor-mediated effects in the isolated cardiac myocytes, we analyzed in the same manner the effects of the β-adrenoceptor agonist isoproterenol at concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. For analysis and comparison of negative inotropic effects, stimulation of cardiac myocytes took place in the presence of verapamil at concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M.

2.3.2. Blocking of the sGC

The effects of the sGC heme-dependent stimulator riociguat and the sGC heme-independent activator cinaciguat were studied in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Sigma-Aldrich, Hamburg, Germany), a selective and potent sGC inhibitor that oxidizes the prosthetic heme of the sGC. For this purpose, cardiac myocytes were pre-incubated with 25 μM ODQ for 30 min (Sandirasegarane and Diamond, 1999), followed by addition of cinaciguat or riociguat – both at a concentration of 10^{-6} M.

2.3.3. Cardiac myocyte stimulation with a membrane permeable cGMP-analog

For further evaluation of cGMP-mediated effects, isolated rat cardiac myocytes were incubated with the membrane-permeable